IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	)	
	)	
Dupret, et al.	)	Group Art Unit: 1637
	)	
Application Number: 09/840,861	)	Examiner: Young J. Kim
	)	
Filed: April 25, 2001	)	
	)	

For: PROCESS FOR IN VITRO CREATION OF RECOMBINANT POLYNUCLEOTIDE SEQUENCES BY ORIENTED LIGATION

SUBMISSION OF ENGLISH LANGUAGE TRANSLATION AND VERIFICATION OF TRANSLATION

MAIL STOP AF
Commissioner for Patents
P.O. Box 1450
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Dear Sir:

Applicant submits herewith an English language translation of the certified copy of the foreign priority document (French Patent Application No. 98/10338), together with a statement declaring that the translation is a true and accurate translation of the original text. Applicant had previously filed the certified copy of the foreign priority document (French Patent Application No. 98/10338) in the above-indicated application on December 29, 2003.

No fee is believed due as a result of this submission. However, if a fee is due upon the filing of this document, please charge such fee to the undersigned's Deposit Account No. 50-0206.

Respectfully submitted,

Dated: July 20, 2005 By: _____

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VERIFICATION OF TRANSLATION

I, Melissa Stanford, a translator with Chillson Translating Service, 3530 Chas Drive, Hampstead, Maryland, 21074, hereby declare as follows:

That I am familiar with the French and English languages;

That I am capable of translating from French to English;

That the translation attached hereto is a true and accurate translation of French Application titled, "PROCESS FOR *IN-VITRO* PRODUCTION OF RECOMBINED POLYNUCLEOTIDE SEQUENCES, SEQUENCE BANKS AND SEQUENCES THAT ARE THUS OBTAINED;"

That all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true;

And further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any registration resulting therefrom.

By Melissa Stanford

Executed this 18 day of May 2005.

Witness Anne Chillson

THE REPUBLIC OF FRANCE

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PATENT

CERTIFICATE OF DESIGN - CERTIFICATE OF ADDITION

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The General Director of the National Institute of Industrial Property certifies that the attached document is a certified true copy of an application for a certificate of industrial property that is filed with the Institute.

Done in Paris on SEPTEMBER 11, 2003

For the General Director of the National

Institute of Industrial Property

Head of the Patent Department

/s/

Martine PLANCHE

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	P071B1246FR	0147036777

2 **APPLICATION Nature of the industrial property title**

Drawing-up of the search report X immediate

The applicant, individual, requires progress payments on the fee X yes

Title of the Invention (200 characters maximum)

PROCESS FOR IN-VITRO PRODUCTION OF RECOMBINED POLYNUCLEOTIDE SEQUENCES, SEQUENCE BANKS AND SEQUENCES THAT ARE THUS OBTAINED

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5 **REDUCTION OF THE FEE LEVEL**

6 **DECLARATION OF PRIORITY OR REQUEST TO TAKE ADVANTAGE OF THE FILING DATE OF A PRIOR APPLICATION**

Country of origin Number Filing Date Nature of the Application

7 **DIVISIONS** prior to this application No. Date No. Date

8 **SIGNATURE OF THE APPLICANT OR AGENT**

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NATIONAL REGISTRATION NUMBER

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TITLE OF THE INVENTION:

PROCESS FOR IN-VITRO PRODUCTION OF RECOMBINED POLYNUCLEOTIDE SEQUENCES, SEQUENCE BANKS AND SEQUENCES THAT ARE THUS OBTAINED.

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January 19, 1999

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921038

/s/

PROCESS FOR *IN-VITRO* PRODUCTION OF RECOMBINED POLYNUCLEOTIDE SEQUENCES, SEQUENCE BANKS AND SEQUENCES THAT ARE THUS OBTAINED.

This invention relates to a method for *in-vitro* production of recombined polynucleotide sequences. The object of the invention is most particularly to generate and then to select polynucleotide sequences that are likely to exhibit advantageous properties relative to reference sequences and are therefore able to impart a new phenotype and/or to produce new proteins.

Different techniques have been developed to promote the *in-vitro* recombination between different polynucleotide sequences; among the latter, it is possible to cite more particularly DNA shuffling (11) and StEP (13), both based on the use of the PCR.

DNA shuffling comprises two stages, the random fragmentation by DNAse I of polynucleotide sequences, and an amplification by PCR in which the fragments that are produced above are used as primers. With each hybridization stage, the change in matrix brings about recombinations with regard to the regions that have homologous sequences. A diagrammatic representation of this method is provided in Figure 1A as an attachment.

StEP consists in mixing different polynucleotide sequences that contain various mutations in the presence of a pair of primers. This mixture is subjected to a PCR-type reaction, in which the stages of hybridization and polymerization are combined in a single stage of very short duration. These conditions allow the hybridization of the primers but reduce the polymerization speed, such that the fragments that are partially synthesized are randomly hybridized onto the polynucleotide sequences that carry the various mutations, thus allowing the recombination. A diagrammatic representation of this method is provided in Figure 1B, attached.

In each of these two methods, the polymerization stage is essential to the recombination

process. Thus, based on selected polymerases, this polymerization stage can generate undesirable further mutations. In addition, starting from a certain number of cycles, the DNA shuffling and the StEP rest on the principle of the hybridization of a "mega-primer" (6) onto a matrix, which probably brings about difficulties of use for polynucleotide sequences whose size is greater than 1.5 Kpb (10). Finally, these two techniques do not make it possible to monitor the rate of recombinations, since the latter are made randomly during successive polymerization stages.

The specific object of this invention is to remedy the drawbacks above by offering a simple method for preparation of recombined polynucleotide sequences, making it possible to generate polynucleotide sequences that can exhibit advantageous properties relative to reference sequences and are therefore able to impart a new phenotype and/or to produce new proteins.

This object is attained thanks to a process for *in-vitro* production of recombined polynucleotide sequences starting from a bank of polynucleotide sequences, characterized in that it comprises the following stages:

a) the fragmentation of an initial bank of double-strand polynucleotide sequences,

b) the denaturation of fragments obtained from stage (a) optionally in the presence of one or more assembly matrices,

c) the hybridization of said fragments in the presence of one or more assembly matrices if the latter is not (are not) present in stage (b),

d) the ligation of said fragments,

e) the cloning of recombined polynucleotide sequences.

The process of the invention can also comprise one or more of the following

stages:

- the repetition of stages (b), (c) and (d) at the end of stage (d),
- the separation of the recombined polynucleotide sequences from the assembly matrix or matrices before stage (e),
- the amplification of the double-strand recombined polynucleotide sequences before cloning stage (e).

The ends of the fragments generated in stage (a) are such that there can be adjacent hybridization of these ends onto the assembly matrix or matrices in stage (c) and ligation of these fragments with one another in stage (d). The polynucleotide sequences of the initial bank should exhibit areas of homology either between one another or with the assembly matrices, making it possible to generate ends of fragments as described above.

An advantageous embodiment of the process of the invention consists in producing stages (c) and (d) simultaneously according to a reaction called RLR for the English expression of "Recombining Ligation Reaction."

In addition to the advantages indicated above, the process of the invention is noteworthy in that it promotes and accelerates the random *in-vitro* recombination of polynucleotide sequences, whereby these polynucleotide sequences can be genes.

The *in-vitro* recombination of polynucleotide sequences of the initial bank by the process of the invention thus makes it possible to obtain a new bank that contains sequences that have accumulated the characteristics of the sequences of the preceding bank. The process of the invention therefore constitutes an *in-vitro* evolution technique.

The process of the invention constitutes an alternative to recombinatory PCR such as that

used in the techniques of DNA shuffling (11) or of StEP (13), since it does not require an *in-vitro* polymerization stage to ensure the recombination. On the contrary, the key stage of the process of the invention is high-temperature ligation stage (d) on an assembly matrix, which ensures a very high degree of accuracy during recombination events.

The fragmentation of these polynucleotide sequences in stage (a) makes it possible to control with precision the desired degree of recombination and the position of recombination points. Thus, the higher the number of fragments generated per sequence, the higher the number of fragments necessary for recomposing a sequence, which brings about a high recombination level. In addition, the nature and the position of the ends of the fragments generated in this embodiment of the process of the invention are known and controlled, which makes it possible:

- to control with precision the zones where the recombination took place, or
- to induce the recombination between polynucleotide sequences, for example genes, if the ends of fragments are created in homology zones between these sequences, or homology zones between these sequences and the assembly matrix or matrices.

The process of the invention can be applied to the orientation of the free-end multi-molecule ligation. In this application, oligonucleotides that are just complementary to end 3' of a fragment and 5' of the adjacent fragment, which allows the adjacent hybridization of these two ends onto the same matrix after the denaturation stage, are used as an assembly matrix in stage b or c. Once hybridized, the ends of the fragments can be ligated together so as to orient the ligation direction of the free-end fragments.

The initial bank of stage (a) can be generated by any method that is known to one skilled

in the art, for example from a savage gene by mutagenesis stages that are directed in succession, by "error-prone" PCR (2), by random chemical mutagenesis, by random *in-vivo* mutagenesis, or by combining genes of close or separate families within the same type or different types so as to use a variety of polynucleotide sequences in the initial bank.

Among these techniques, the invention considers more particularly a process in which the initial bank of double-strand polynucleotide sequences is obtained by a polymerization chain reaction that is carried out under conditions that make it possible to create random mutations.

According to a preferred embodiment of the process of the invention, stage (a) consists in subjecting the initial bank to hydrolysis by the action of one or more restriction enzymes.

To increase the degree of recombination generated by the process of the invention, it is sufficient to increase the number of restriction fragments by using restriction enzymes that have a large number of cutting sites on the polynucleotide sequences of the initial bank, or by combining several restriction enzymes. In the case of use of a thermostable ligase, the limit is set by the size of the smallest fragment thus generated, for example greater than or equal to 40 pb, so as to preserve a hybridization temperature that is compatible with that of ligation stage (d), which is generally on the order of 65°C.

Stage (a) can also be carried out by generating a bank of fragments by random treatment with the DNAse I of an initial bank of double-strand polynucleotide sequences that are partially heterologous. This implementation of the process of the invention would have the particular feature of making possible the use of DNAse I fragments such as matrices for one another, for hybridization during stage (c) or the simultaneous RLR reaction of stages (c) and (d).

A similar implementation of the process of the invention can be carried out by combining

with stage (b) at least two banks of separate fragments generated separately in stage (a) from the same initial bank by a treatment with different enzymes. The use of such banks no longer requires the use of a consensus matrix during stage (b) or (c), since the fragments can be hybridized onto one another.

The fragments of stage (a) of the process of the invention can also be generated by PCR reactions conducted on the polynucleotide sequences of the initial bank. Two solutions are conceivable. In a first case, the oligonucleotides can be designed so as to generate fragments whose ends are adjacent all along the assembly sequence. In a second case, the oligonucleotides are designed so as to generate fragments that have common sequences, whereby these fragments can be used as an assembly matrix for one another in stage (b) or in stage (c).

The assembly matrix in stage (b) or (c) is, for example, a polynucleotide sequence that is obtained from the initial bank or a consensus sequence of said bank, single- or double-strand. In the case where the assembly matrix is incorporated directly into stage (c) of the invention, this matrix should be in single-strand form.

According to a variant of the process of the invention, the assembly matrices of stage (b) or (c) consist of oligonucleotides.

According to a particular embodiment of the process of the invention, single- or double-strand oligonucleotides of variable length are added at stage (c) in addition to the matrix. These oligonucleotides are designed to be able to be substituted for a portion of the fragments in stage (c), actually, their sequence is such that:

- if they are completely homologous with the sequence of the fragment that they are replacing, they promote certain combinations, or

- if they are partially heterologous with the sequence of the fragment that they are replacing, they introduce one or more further directed mutations.

Before stage (e) of the process of the invention, it is possible to separate the recombined polynucleotide sequences from the assembly matrix thanks to a marker that is present on the assembly matrix or on the recombined polynucleotide sequences. It is actually possible to mark each strand of the matrix according to techniques known by one skilled in the art. For example, the marker of the assembly matrix can be a hapten, and the recombined polynucleotide sequences of the assembly matrix are separated by techniques that are known to one skilled in the art, such as, for example, an anti-hapten antibody that is attached to a substrate or a biotin-streptavidin reaction, if the hapten is a biotin marker.

Other techniques may be used to separate the recombined polynucleotide sequences from the assembly matrix. The assembly matrix can also be prepared specifically so as to facilitate its elimination at the end of the process of the invention. It can thus be synthesized by PCR amplification using methylated dATP, which allows its degradation by the *Dpn* I restriction endonuclease. In this case, the recombined polynucleotide sequences should not contain methylated dATP. The matrix may also have been prepared by PCR amplification by using dUTP, which allows its degradation by treatment with a uracyl-DNA-glycosylase. Conversely, it is possible to protect the recombined polynucleotide sequences by amplifying them by selective PCR with oligonucleotides that carry phosphorothioate groups at 5'. Treatment with an exonuclease then makes it possible specifically to degrade the assembly matrix.

As indicated above, the process of the invention can comprise, before cloning stage (e), a stage for amplification of recombined polynucleotide sequences. Any amplification technique is

acceptable, in particular an amplification by PCR. One of the simplest consists in producing a PCR that makes it possible to amplify specifically the recombined polynucleotide sequences thanks to primers that can be hybridized only onto the ends of recombined sequences. The PCR products are then cloned so that they can be characterized.

The invention also relates to a process for generating polynucleotide sequences that are likely to have advantageous properties compared to reference sequences. This process consists in producing recombined polynucleotide sequences according to the process that is described above, then in screening by any suitable means the clones that are obtained for selecting those that comprise recombined polynucleotide sequences that have advantageous properties relative to reference sequences. For example, advantageous properties are defined as the thermostability of an enzyme or its capacity to be able to operate under conditions of pH or temperature or saline concentration that are more suitable to an enzymatic process than the reference proteins that are usually used for said process. By way of example of such a process, it is possible to cite an industrial process for desizing textile fibers or for bleaching paper pastes or for producing aromas in the dairy industry.

According to an advantageous embodiment of this application of the process of the invention, the initial bank can therefore be the result of a screen that has made it possible to select by any suitable means the polynucleotide sequences that exhibit advantageous properties relative to reference sequences. The thus selected sequences constitute an initial limited bank.

It is also possible, however, to start from an initial unlimited bank so as to preserve the representative quality of the properties contained in this bank.

The sequences that code for the proteins that have advantageous properties relative to the

reference proteins are then selected, by *in-vivo* and *in-vitro* screenings, and can be used in constituting a new bank for an optional reiteration of the process of the invention.

Among the screening techniques that can be applied to each of the clones of stage (e), the *in-vitro* screening techniques offer the advantage of eliminating problems of cellular physiology and all the drawbacks that are linked to the *in-vivo* expression cloning. In addition, this type of screening is easy to automate, which makes it possible to screen a high number of recombined polynucleotide sequences.

The invention also relates to a recombined polynucleotide sequence that is obtained by a process according to the invention, as well as a vector that contains such a recombined polynucleotide sequence, a cellular host that is transformed by a recombined polynucleotide sequence or a vector of the invention, as well as a protein that is coded by this recombined polynucleotide sequence. The invention also comprises the corresponding banks of recombined polynucleotide sequences, vectors, cellular hosts or proteins.

Other advantages and characteristics of the invention will emerge from the following embodiments of the invention that refer to the accompanying drawings, in which:

Figure 1 is a diagrammatic representation of the processes of the prior art corresponding respectively to DNA shuffling (Figure 1A) and to StEP (Figure 1B).

Figure 2 is a diagrammatic representation of an embodiment of the process of the invention and of some of its variants and application.

Figure 3 represents the positions of the ten mutation zones (*Pvu* II and *Pst* I) carried by each mutant of the *ponB* gene that is used for the embodiments of the invention.

Figure 4 represents the position of the primers that are used relative to the sequence of the

ponB gene.

Figure 5 represents the migration of the products of the RLR and PCR reactions of the products of these RLR reactions to agarose gel.

Figure 6 represents the position of the mutations relative to the restriction fragments.

I – PRINCIPLE.

The process of the invention was used starting from a bank of mutants of the *ponB* gene, coding for the PBP1b of *E. coli* (1). Ten mutants of this gene were used. The gene sequence of each mutant differs from that of the savage gene by a non-homologous zone of thirteen to sixteen bases consisting of the substitution of five initial codons by five alanine codons according to the technique described by Lafèvre *et al.* (8).

The substitution carried by every mutant is characterized by the presence of a single site of the *Pvu* II restriction enzyme framed by two sites of the *Pst* I enzyme, which make it possible to distinguish the mutants from one another by their digestion profile with these restriction endonucleases. Figure 3 shows the positions of ten mutation zones (*Pvu* II and *Pst* I) that are carried by every mutant.

After amplification by PCR of the genes of the ten mutants, the PCR products were purified, mixed in an equimolar amount to constitute the initial bank. The polynucleotide sequences of this bank were digested by restriction enzymes *Hinf* I and *Bsa* I, so as to generate restriction fragment banks. The restriction fragments were then incubated with the savage matrix, in different amounts, in the presence of a thermostable ligase. After several cycles of denaturation/hybridization/ligation, a fraction of this reaction mixture was used to carry out a

PCR amplification with a pair of specific primers of ends 5' and 3' of the genes of mutants and non-specific genes of ends 5' and 3' of the savage matrix. The amplification product was cloned, and the clones obtained were analyzed for their digestion profile with restriction endonuclease *Pvu* II or *Pst* I. The profiles obtained made it possible to determine which fragment(s) of the mutants was (were) able to be recombined with the others to reconstitute an entire gene.

II – MATERIAL.

1) Colonies and Plasmids.

The MC1061 colony (F^- *araD*139, Δ (*ara-leu*)$_{7696}$, *galE15, galK16,* Δ *(lac)X*74, *rpsL* (Str^R), *mcrA mcrB1, hsdR2* ($r_k^- m_k^+$)) is derived from *Escherichia coli* K12.

The pARAPONB vector is obtained from the pARA13 vector (3) in which the *ponB* gene that carries a thrombin cutting site (9) was introduced between the *Nco* I and *Nar* I restriction sites. The pET26b+ vector is part of the family of pET vectors developed by Studier and Moffatt (12) and marketed by the NOVAGEN Company.

2) Oligonucleotides.

The oligonucleotides were synthesized by the ISOPRIM Company (Toulouse). The sequences of the oligonucleotides are reported in Table I below.

Table I

Oligo N	5' ACTGACTACCATGGCCGGGAATGACCGCGAGCC 3'
Oligo E	5' CCGCGGTGGAGCGAATTCTAATTACTACCAAACATATCC 3'
Oligo M1	5' GCGCCTGAATATTGCGGAGAAAAAGC 3'
Oligo M2	5' ACAACCAGATGAAAAGAAAGGGTTAATATC 3'
Oligo A1	5' ACTGACTACCATGGCC 3'
Oligo A2	5' CCGCGGTGGAGCGAATTC 3'

3) Reagents.

The enzymes of restrictions and modifications cited in Table II below were used according to the recommendations of the suppliers.

Table II

Enzyme	Concentration	Supplier
NcoI	10 U/µl	NEB
PstI	20 U/µl	NEB
Eco RI	20 U/µl	NEB
Bsa I	5 U/µl	New England Biolabs
Hinf I	10 U/µl	New England Biolabs
Pvu II	10 U/µl	New England Biolabs
T4 DNA ligase	400 U/µl	New England Biolabs
Taq DNA polymerase	5 U/µl	PROMEGA
AMPLIGASE	100 U/µl	EPICENTRE

The buffers that are used are reported in Table III below.

Table III

Buffers	Composition
T	10 mmol of Tris HCl, pH 8.0
20X Polymerization	100 mmol of Tris HCl, pH 8.3, 15 mmol of $MgCl_2$, 500 mmol of KCl, 1.0% Triton X100®
10X Restriction A	500 mmol of NaCl, 100 mmol of Tris HCl, pH 7.9, 100 mmol of $MgCl_2$, 10 mmol of DTT
10X Restriction B	1 M NaCl, 500 mmol of Tris HCl, pH 7.9, 100 mmol of $MgCl_2$, 10 mmol of DTT
10X Restriction C	500 mmol of NaCl, 1 M Tris HCl, pH 7.5, 100 mmol of $MgCl_2$, 0.25% Triton X100®
10X AMPLIGASE	200 mmol of Tris HCl, pH 8.3, 250 mmol of KCl, 100 mmol of $MgCl_2$, 5 mmol of DNA, 0.1% Triton X100®
10X LIGATION	500 mmol of Tris HCl, pH 7.5, 100 mmol of $MgCl_2$, 100 mmol of DTT, 10 mmol of ATP, 250 μg/ml of BSA

III – PREPARATION OF THE MATRIX.

The savage *ponB* gene was amplified by a PCR reaction stage by using the M1 and M2 oligonucleotides as primers (Fig. 4). Five PCR reactions were prepared by adding 50 ng of the pPONBPBR plasmid that carries savage gene (7) with a mixture that contains 10 µl of polymerization buffer, 10 µl of 2 mmol dNTPs, 20 pmol of each M1 and M2 oligonucleotide and 5U of Taq DNA polymerase, in a final volume of 100 µl. These mixtures were incubated in a Perkin-Elmer 9600 thermal cycler according to the following program: (94°C – 2 minutes) – (94°C, 15 seconds - 60°C, 30 seconds - 72°C, 1 minute) x 29 cycles – (72°C – 3 minutes).

The product of the five PCR was mixed and deposited on a 1% TBE agarose gel. After migration and coloration with the ethidium bromide of the gel, the band at 2651 pb, corresponding to the amplification product of the *ponB* gene framed by two fragments of 26 pb and 90 pb respectively, was visualized by ultraviolet trans-illumination and cut with a scalpel to be purified with the Quiaquick (QIAGEN) system. The complete DNA thus purified was eluted in 120 µl of T buffer. The concentration of this DNA was evaluated by a spectrophotometric dosage at 260 nm, at 100 ng/µl.

IV – PREPARATION OF THE BANK.

1) Amplification of Mutant Genes.

The genes of the ten mutants were amplified separately via a PCR reaction by using the N and E oligonucleotides. These oligonucleotides respectively introduce the *Nco* I and *Eco* RI restriction sites, making it possible to clone the products that are obtained with these two sites.

Each PCR reaction was prepared by adding 50 ng of the plasmid that carries the mutant

gene with a mixture that contains 10 µl of polymerization buffer, 10 µl of 2 mmol dNTPs, 20 pmol of each N and E oligonucleotide, and 5U of Taq DNA polymerase, in a final volume of 100 µl. This mixture was incubated in a Perkin-Elmer 9600 thermal cycler according to the following program: (94°C – 2 minutes) – (94°C, 15 seconds - 60°C, 30 seconds - 72°C, 1 minute) x 29 cycles – (72°C – 3 minutes).

The specificity of the gene amplification was verified by restriction profile with the *Pvu* II endonuclease by incubating 5 µl of each PCR product for 1 hour at 37°C in a mixture that contains 3 µl of restriction buffer A and 5U of the *Pvu* II enzyme in a final volume of 30 µl. 15 µl of this digestion reaction was deposited on a 1% TBE agarose gel. After migration and coloration with ethidium bromide, the gel was exposed to ultraviolet. The visualization of the restriction fragments made it possible to confirm the specificity of the gene amplification of each mutant gene.

Concurrently, 3 µl of each PCR reaction was deposited on a 1% TBE agarose gel. After migration, the gel was treated as above. The intensity of each band made it possible to estimate that the gene amplifications had had the same yield.

2) Creation of Restriction Fragment Banks.

50 µl of each of the ten PCR was mixed and deposited on a 1% TBE agarose gel. After migration and coloration with ethidium bromide, the band at 2572 pb, corresponding to the product for amplification of the genes of the ten mutants, was cut with a scalpel and purified with the Quiaquick (QIAGEN) system. The entire DNA thus purified was eluted in 120 µl of T buffer. The concentration of this DNA was evaluated by a spectrophotometric dosage at 260 nm,

at 100 ng/µl.

To generate the restriction fragment banks, 100 µl of this DNA was incubated for one hour at 50°C in a mixture that contains 12 µl of restriction buffer B, 1.2 µl of BSA (with 10 mg/ml), 25 U of the *Bsa* I enzyme, and 4 µl of water. Then, 2 µl of restriction buffer B, 2 µl of BSA (with 1 mg/ml), 50 U of the *Hinf* I enzyme and 11.5 µl of water were added to the mixture, which was incubated for one hour at 37°C. The entire digestion mixture was purified on a QIAquick (QIAGEN) column and eluted with 30 µl of buffer T. 1 µl of this eluant was deposited on 1% TBE agarose gel to verify that the digestion had been total and that it generated 6 restriction fragments, and consequently six fragment banks, of 590 pb, 500 pb, 472 pb, 438 pb, 298 pb, and 274 pb. The concentration of this DNA was evaluated (by a spectrophotometric dosage at 260 nm) at 250 ng/µl.

V – RLR (Recombining Ligation Reaction).

The RLR (Recombining Ligation Reaction) reaction was carried out by incubating determined amounts of *Hinf* I – *Bsa* I restriction fragments of genes of the ten mutants with the complete matrix (i.e., the savage *ponB* gene), in the presence of a thermostable DNA ligase. Table IV below reports the composition of the RLR mixtures.

Table IV

	RLR 1	RLR 2	RLR 3	RLR 4	T-
Hinf I – *Bsa* I Fragments of the Ten Mutants (100 ng/μl)	0.5 μl	1 μl	2 μl	5 μl	5 μl
Savage *ponB* Matrix (100 ng/μl)	0.6 μl	1.2 μl	2.4 μl	6 μl	6 μl
10X AMPLIGASE Buffer	2 μl	2 μl	2 μl	2 μl	2 μl
AMPLIGASE (25 U/μl)	1 μl	1 μl	1 μl	1 μl	-
H_2O	Sufficient quantity for 20μl	Sufficient quantity for 20μl	Sufficient quantity for 20μl	Sufficient quantity for 20μl	Sufficient quantity for 20μl

The negative control is identical to the RLR4 reaction, but it does not contain thermostable DNA ligase. These different mixtures were recovered from a drop of mineral oil and incubated in a Perkin-Elmer 9600 thermal cycler in 200 μl microtubes according to the following program: (94°C, 5 minutes) – (94°C, 1 minute - 65°C, 4 minutes) x 35 cycles.

10 μl of each RLR reaction was then added to a PCR reaction mixture that contains 10 μl of polymerization buffer, 10 μl of 2 mmol dNTPs, 40 pmol of each A1 and A2 oligonucleotide, and 5 U of Taq DNA polymerase in a final volume of 100 μl. This mixture was incubated in a Perkin-Elmer 9600 thermal cycler according to the following program: (94°C, 5 minutes) – (94°C, 30 seconds - 46°C, 30 seconds - 72°C, 1 minute) x 29 cycles – (72°C, 2 minutes). This PCR reaction made it possible to amplify specifically the ligation products that are formed during the RLR reaction, without amplifying the matrix, then the A1 and A2 oligonucleotides cannot be hybridized onto the latter, as shown in Figure 4.

5 μl of each RLR reaction and 10 μl of each of the preceding PCR reactions was deposited on a 1% TBE agarose gel. After coloration with ethidium bromide, the gel was exposed to ultraviolet, as shown in Figure 5.

The analysis of this gel reveals that only the RLR4 reaction contains, as the negative control, restriction fragments that are still visible (tracks 4 and 5).

The absence of the PCR product for the negative control (track 10) reveals not only that the PCR reaction is specific (no amplification of the complete matrix) but also that the restriction fragments that are present in the mixture cannot be substituted with primers to generate a contaminating PCR product under the selected conditions. Concurrently, the presence of a single band with about 2500 pb on tracks 6, 7 and 8 demonstrates that an RLR product could be amplified by PCR for the RLR1, 2 and 3 reactions. These three RLR reactions were therefore allowed to reconstitute one or more complete genes from six banks of restriction fragments.

VI – ANALYSIS OF PRODUCTS FOR AMPLIFICATION OF THE RLR REACTIONS.

1) Cloning.

The PCR amplification products of the RLR 1, 2 and 3 reactions were purified with the Wizard PCR Preps System (PROMEGA) and eluted in 45 µl of buffer T. 6 µl of each purified PCR was incubated for 1 hour at 37°C in a mixture that contains 3 µl of restriction buffer C, 3 µl of BSA (at 1 mg/ml), 20 U of the *Eco* RI enzyme, 10 U of the *Nco* I enzyme, and 15 µl of water.

Concurrently, two vectors (pARAPONB and pET26b+) were prepared for the cloning. These vectors were linearized by incubating 3 µg of these plasmids for 2 hours at 37°C, in a mixture that contains 3 µl of restriction buffer C, 3 µl of BSA (at 1 mg/ml), 20 U of the *Eco* RI enzyme, 10 U of the *Nco* I enzyme and 19 µl of water.

The linearized vectors as well as the digested PCR were purified on 1% TBE agarose gel

with the QIAquick (QUIAGEN) System. Each vector or each digested PCR was eluted in 30 µl of buffer T.

The ligation of each PCR digested with one or the other of the vectors was carried out according to the conditions that are described in Table V below, and incubated at 16°C for 16 hours.

Table V

	Ligation with the pARAPONB Vector				Ligation with the pET26b+ Vector			
	LpAR1	LpAR2	LpAR3	TlpAR	LpET1	LpET2	LpET3	TLpET
PCR Amplification of the digested RLR 1 *Nco* I – *Eco* – RI	4 µl	-	-	-	4 µl	-	-	-
PCR Amplification of the digested RLR 2 *Nco* I - *Eco* RI	-	4 µl	-	-	-	4 µl	-	-
PCR Amplification of the digested RLR 3 *Nco* I – *Eco* RI	-	-	4 µl	-	-	-	4 µl	-
Nco I- *Eco* RI -digested pARAPONB Vector	1 µl	1 µl	1 µl	1 µl	-	-	-	-
Nco I- *Eco* RI digested pET26b+	-	-	-	-	1 µl	1 µl	1 µl	1 µl

	Ligation with the pARAPONB Vector				Ligation with the pET26b+ Vector			
	LpAR1	LpAR2	LpAR3	TlpAR	LpET1	LpET2	LpET3	TLpET
Vector								
Ligation Buffer	2 µl	2 µl	2 µl	2 µl	2 µl	2 µl	2 µl	2 µl
Ligase	1 µl	1 µl	1 µl	1 µl	1 µl	1 µl	1 µl	1 µl
H_2O	12 µl	12 µl	12 µl	16 µl	12 µl	12 µl	12 µl	16 µl

200 µl of chemocompetent MC1061 cells (4) were transformed with 10 µl of each ligation by a thermal shock (5), and the thus transformed cells were smeared on a selection medium.

No clone was obtained after transformation of the TL pAR and TL pET ligation controls, thus indicating that *Nco* I – *Eco* RI linearized pARAPONB and pET26b+ vectors cannot undergo intramolecular ligation.

2) Screening by PCR.

A first screening of clones obtained after transformation of ligations with the pARAPONB vector was carried out by PCR. 42 colonies, 14 of each LpAR1, LpAR2 and LpAR3 ligation, were resuspended individually in a PCR mixture containing 5 µl of polymerization buffer, 40 pmol of each A1 and A2 oligonucleotide, 5 µl of 2 mmol dNTPs and 5U of Taq DNA polymerase in a final volume of 50 µl. A negative control was constituted by adding to the PCR mixture 50 ng of pBR322 plasmid instead of a colony. These 43 tubes were incubated in a Perkin-Elmer 9600 thermal cycler according to the following program: (94°C, 5

minutes) – (94°C, 30 seconds - 46°C, 30 seconds - 72°C, 1 minute) x 29 cycles – (72°C, 2 minutes). 5 µl of each of these PCR reactions was then incubated for 1 hour at 37°C in a mixture containing 2 µl of restriction buffer A, 2 µl of BSA (at 1 mg/ml) and 5 U of *Pvu* II restriction enzyme in a final volume of 20 µl.

10 µl of each of these digestions was deposited on a 1% TBE agarose gel concurrently with 5 µl of each non-digested PCR (which makes it possible not to combine possible non-specific bands of the PCR with a fragment that is obtained by restriction digestion).

After migration and coloration with ethidium bromide of this gel, the bands that are obtained from the digestion by the *Pvu* II enzyme were analyzed so as to determine what fragment(s) of the initial mutants were associated with the others to reconstitute an entire gene. This screening reveals the presence of 27 genes that support a mutation, 7 genes that support two mutations, and 8 genes that no longer support mutation.

3) Screening by Mini-Preparation of Plasmid DNA.

The second screening was carried out by performing an extraction from plasmid DNA (5) of 21 clones obtained from the transformation of ligations with the pET26b+ vector (7 clones of each ligation). 5 µl of the thus obtained plasmid DNA for each clone was incubated for 1 hour at 37°C in the presence of a mixture that contains 1 µl of restriction buffer C, 6 U of the *Pst* I enzyme, 3 U of the *Nco* I enzyme and 6 U of the *Eco* RI enzyme in a final volume of 10 µl. 5 µl of each of these digestions was deposited on a 1% TBE agarose gel. After migration and coloration with ethidium bromide of this gel, the bands that are obtained from the digestion by the *Pst* I enzyme were analyzed so as to determine what fragment(s) of the initial mutants were

associated with the others to reconstitute an entire gene. This screening reveals the presence of 13 genes that support a mutation, 5 genes that support two mutations and 3 genes that no longer support mutation.

4) Statistical Analysis of Recombinations.

Based on the position of each mutation relative to the cutting sites of the *Hinf* I and *Bsa* I enzymes, as shown in Figure 6, it is possible to calculate the probability of obtaining the creation of a gene that carries 0, 1, 2, 3 or 4 of the mutations of the initial genes during the RLR reaction.

Thus, by considering that the RLR reaction is totally random, probabilities P are as follows:

$$P(0 \text{ mutation}) = \prod_{i=6}^{9} \left(\frac{i}{10}\right) = 30,24\%$$

$$P(1 \text{ mutation}) = \sum_{n=1}^{4} \left[\frac{n}{10-n} \prod_{i=1}^{4} \left(\frac{10-i}{10}\right)\right] = 44,04\%$$

$$P(2 \text{ mutations}) = \sum_{n=1}^{4} \left[\sum_{a=1}^{4-n} \left(\frac{10-a}{a}\right)\left(\frac{10-(a+n)}{a+n}\right) \prod_{i=1}^{4} \left(\frac{i}{10}\right)\right] = 21,44$$

$$P(3 \text{ mutations}) = \sum_{n=1}^{4} \left[\left(\frac{10-n}{n}\right) \prod_{i=1}^{4} \left(\frac{i}{10}\right)\right] = 4,04\%$$

$$P(4 \text{ mutations}) = \prod_{i=1}^{4} \left(\frac{i}{10}\right) = 0,24\%$$

The two screenings that are carried out provide results that are close to these statistical

forecasts, as reported in Table VI below, thus indicating that the RLR reaction is almost random. A slightly larger proportion of genes that support a mutation, to the detriment of the genes that support zero mutation, is observed. This phenomenon could be attributed to a low toxicity of the *ponB* gene already observed and to the slight shift of the pARAPONB and pET26b+ expression vectors that would promote the selection of genes that support an inactivating mutation.

Table VI

%	0 Mutation	1 Mutation	2 Mutations	3 Mutations	4 Mutations
Statistics	30.24	44.04	21.44	4.04	0.24
PCR Screening	21	63	16	0	0
Mini-preparation Screening	14	62	24	0	0

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CLAIMS

1) Process for *in-vitro* production of recombined polynucleotide sequences starting from a bank of polynucleotide sequences, characterized in that it comprises the following stages:

a) the fragmentation of an initial bank of double-strand polynucleotide sequences,

b) the denaturation of fragments obtained from stage (a) optionally in the presence of one or more assembly matrices,

c) the hybridization of said fragments in the presence of one or more assembly matrices if the latter is not (are not) present in stage (b),

d) the ligation of said fragments,

e) the cloning of the recombined polynucleotide sequences.

2) Process according to claim 1, wherein it comprises the repetition of stages (b), (c) and (d) at the end of stage (d).

3) Process according to one of claims 1 or 2, wherein it comprises the separation of the recombined polynucleotide sequences of the assembly matrix or matrices before stage (e).

4) Process according to one of claims 1 to 3, wherein it comprises the amplification of the double-strand recombined polynucleotide sequences before cloning stage (e).

5) Process according to any of claims 1 to 4, wherein the ends of the fragments generated in stage (a) are such that there can be adjacent hybridization of these ends onto the assembly matrix or matrices in stage (c) and ligation of these fragments with one another in stage (d).

6) Process according to any of the preceding claims, wherein the polynucleotide sequences of the initial bank have homology zones either between one another or with the assembly matrices, making it possible to generate ends of fragments that make possible the

adjacent hybridization of these ends onto the assembly matrix or matrices in stage (c) and ligation of these fragments with one another in stage (d).

7) Process according to any of the preceding claims, wherein stages (c) and (d) are carried out simultaneously.

8) Process according to any of the preceding claims, wherein the degree of recombination and the position of the recombination points are controlled by the fragmentation of stage (a).

9) Process according to any of the preceding claims, wherein the initial bank of stage (a) is generated from a savage gene by mutagenesis stages that are directed in succession by random mutagenesis, or by combining genes of close or separate families within the same type or of different types so as to use a variety of polynucleotide sequences in the initial bank.

10) Process according to any of the preceding claims, wherein stage (a) consists in subjecting the initial bank to hydrolysis by the action of one or more restriction enzymes.

11) Process according to any of the preceding claims, wherein stage (a) consists in subjecting the initial bank to hydrolysis by action of restriction enzymes that have a large number of cutting sites on the polynucleotide sequences of the initial bank or by combining several restriction enzymes.

12) Process according to any of the preceding claims, wherein in stage (a), fragments are prepared whose size is greater than or equal to 40 pb.

13) Process according to any of the preceding claims, wherein stage (a) consists in generating a bank of fragments by random treatment with the Dnase I of the initial bank of double-strand polynucleotide sequences.

14) Process according to any of the preceding claims, wherein stage (b) is carried out by

combining at least two banks of separate fragments that are generated separately in stage (a) from the same initial bank by a treatment with different enzymes.

15) Process according to any of the preceding claims, wherein the fragments that are generated in stage (a) are generated by PCR starting from (a) polynucleotide sequence(s) of the initial bank.

16) Process according to one of claims 13 to 15, wherein the fragments that are obtained in stage (a) are used as an assembly matrix for one another in stage (b) or in stage (c).

17) Process according to any of claims 1 to 15, wherein the assembly matrix of stage (b) or (c) of the process is a polynucleotide sequence that is obtained from the initial bank or a consensus sequence of said single- or double-strand bank.

18) Process according to any of claims 1 to 15, wherein the assembly matrices of stage (b) or (c) of the process consist of oligonucleotides.

19) Process according to any of claims 1 to 18, wherein stage (c) is carried out in the presence of single- or double-strand oligonucleotides of variable length.

20) Process for *in-vitro* production of recombined polynucleotide sequences that exhibit advantageous properties relative to reference sequences, wherein a process is carried out according to any of claims 1 to 19, then wherein the clones that are obtained are screened by any suitable means to select those that comprise polynucleotide sequences that exhibit advantageous properties relative to reference sequences.

21) Process according to claim 20, wherein the screening of the clones is carried out *in vitro*.

22) A recombined polynucleotide sequence that is obtained by the process of the

invention according to any of claims 1 to 21.

23) A vector that contains a recombined polynucleotide sequence according to claim 22.

24) A cellular host that is transformed by a recombined polynucleotide sequence according to claim 22 or by a vector according to claim 23.

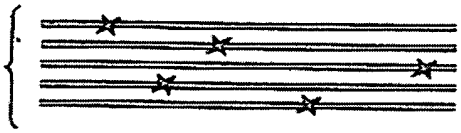
25) A protein that is coded by a recombined polynucleotide sequence according to claim 22.

26) A bank that consists of recombined polynucleotide sequences according to claim 22, or vectors according to claim 23, or cellular hosts that are transformed according to claim 24, or proteins according to claim 25.

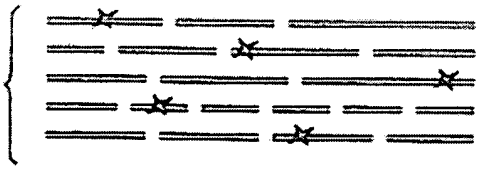
PROCESS FOR *IN-VITRO* PRODUCTION OF RECOMBINED POLYNUCLEOTIDE SEQUENCES, SEQUENCE BANKS AND SEQUENCES THAT ARE THUS OBTAINED.

This invention relates to a method for *in-vitro* production of recombined polynucleotide sequences that comprise the following stages: (a) the fragmentation of an initial bank of double-strand polynucleotide sequences, (b) the denaturation of fragments obtained from stage (a) optionally in the presence of one or more assembly matrices, (c) the hybridization of said fragments in the presence of one or more assembly matrices if the latter is not (are not) present in stage (b), (d) the ligation of said fragments, and (e) the cloning of recombined polynucleotide sequences.

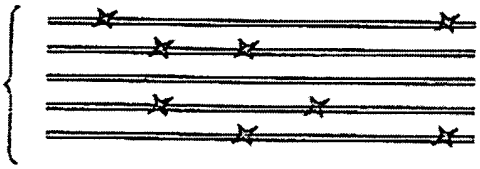
Figure 1 A



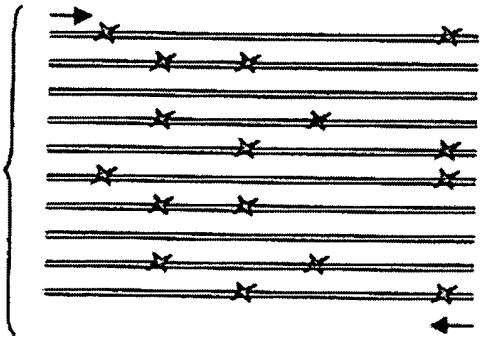
Family of Genes



DNAse I Fragmentation



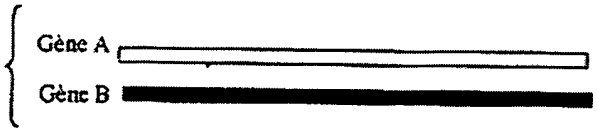
PCR without Primers



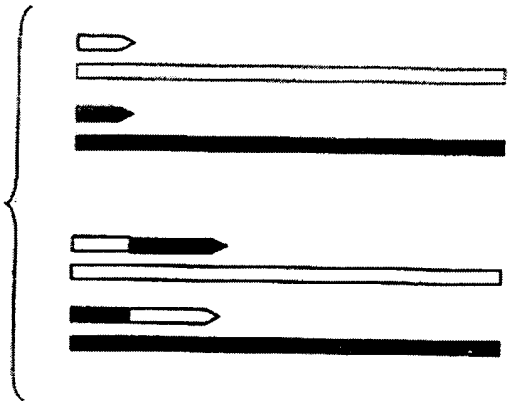
PCR with Primers

Figure 1 B

(Process carried out in a double strand, but illustrated here on a single strand)



Family of Genes

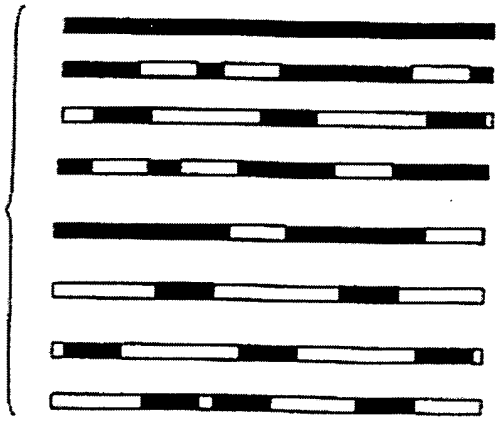


Short Cycles of

Denaturation,

Hybridization,

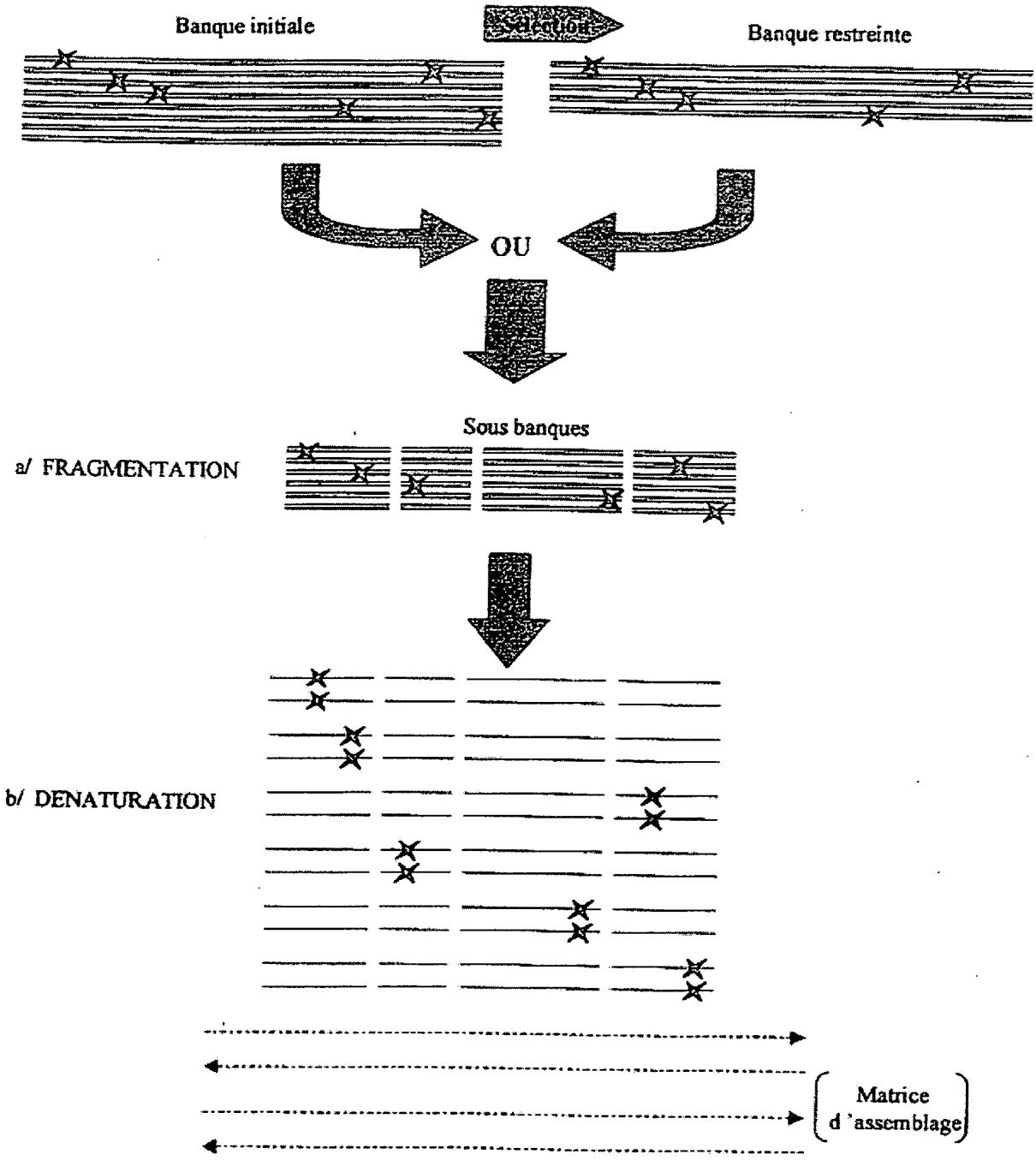
Polymerization



Family of Recombined

Genes

Fig. 2


Banque initiale
Sélection
Banque restreinte
OU
Sous banques
a/ FRAGMENTATION
b/ DENATURATION
Matrice
d 'assemblage

[Key to Figure 2:]

Banque initiale = Initial Bank

Sélection = Selection

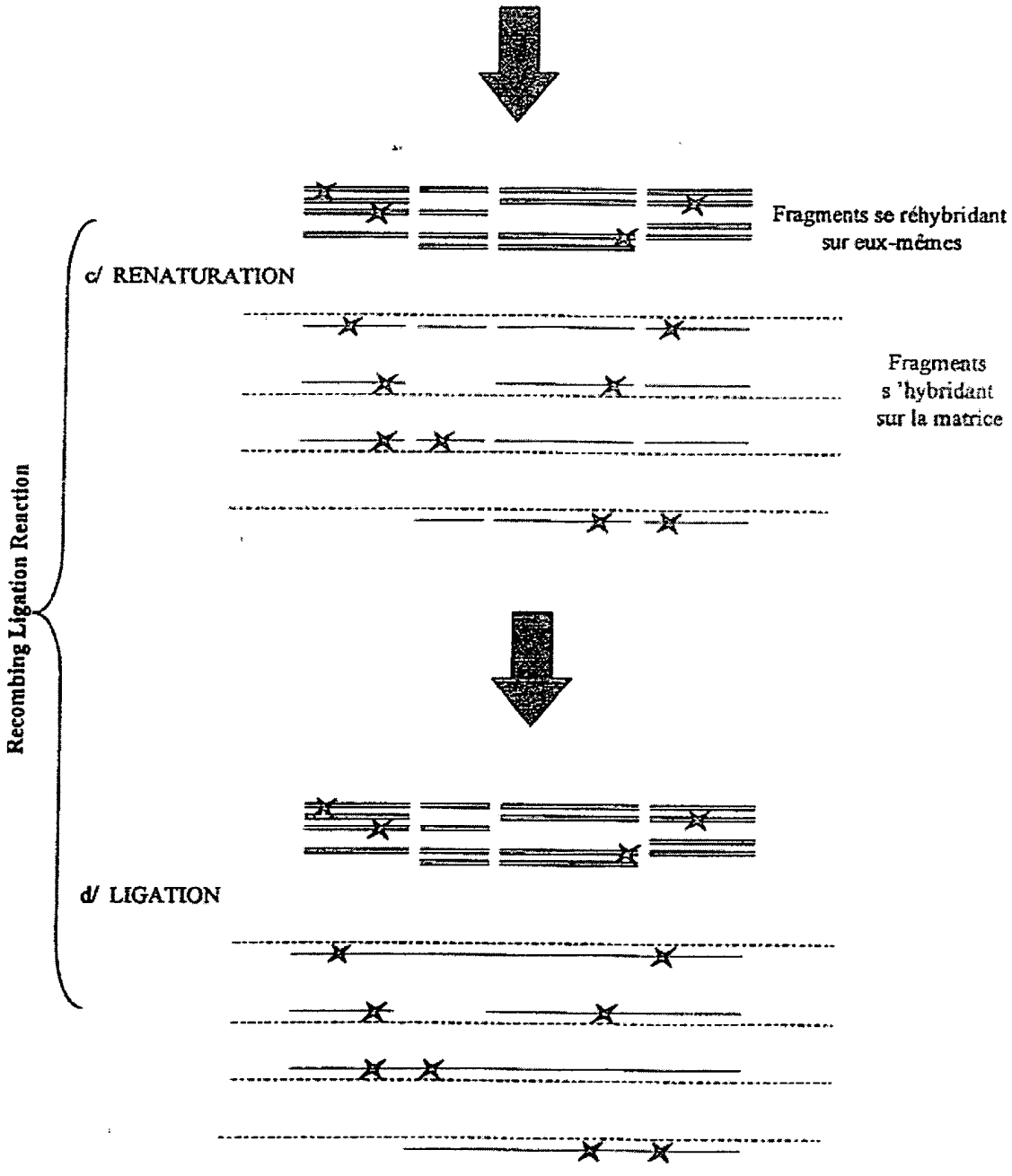
Banque restreinte = Limited Bank

OU = OR

Sous banques = Under Banks

Matrice d'assemblage = Assembly Matrix

Fig. 2 Continuation 1

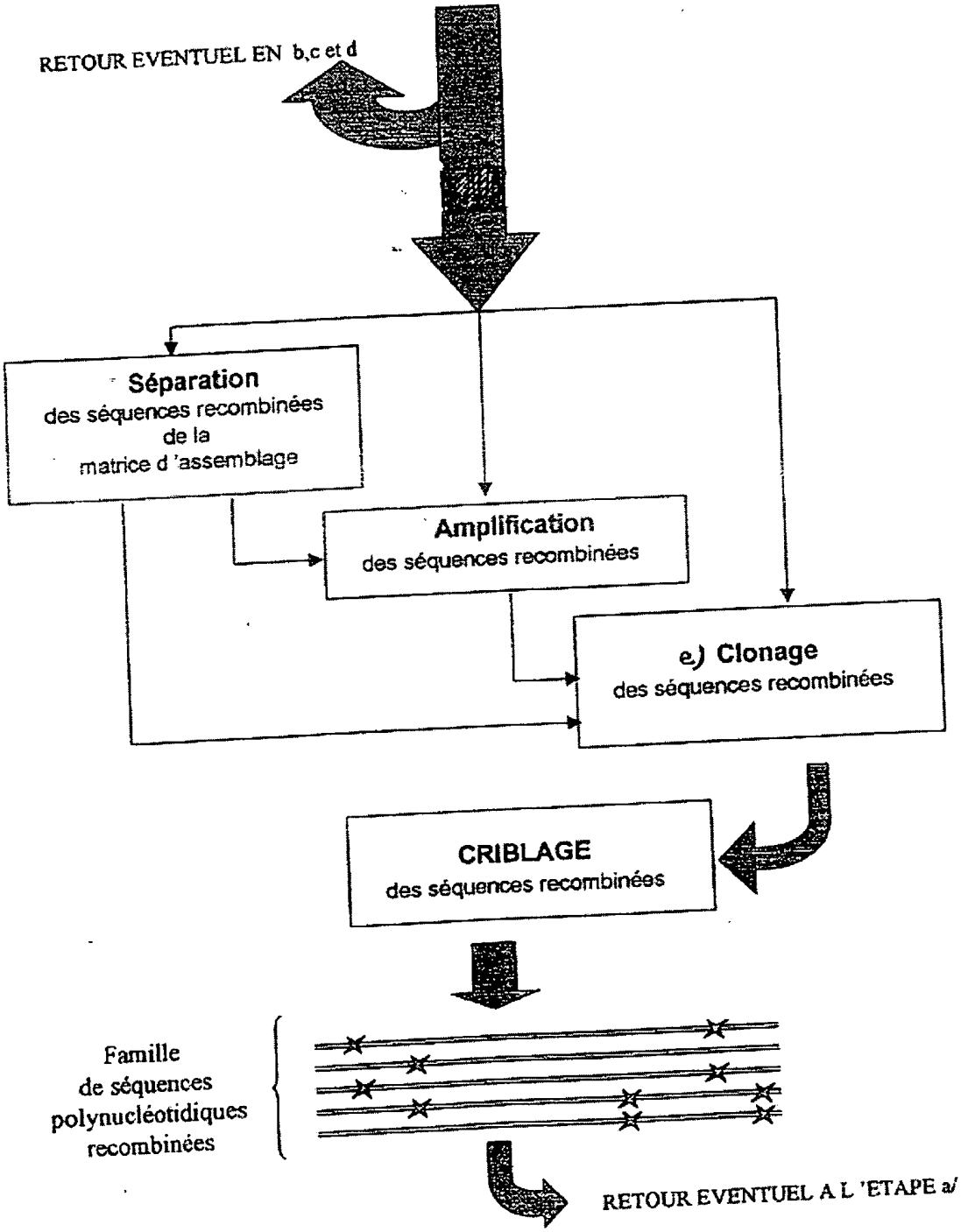


[Key to Figure 2, Continuation 1:]

Fragments se réhybridant sur eux-mêmes = Fragments that are rehybridized onto themselves

Fragments s'hybridant sur la matrice = Fragments that are hybridized onto the matrix

Figure 2, Continuation 2



[Key to Figure 2, Continuation 2]

RETOUR EVENTUEL EN b, c et d = POSSIBLE RETURN TO b, c and d

Séparation des séquences recombinées de la matrice d'assemblage = Separation of the recombined sequences of the assembly matrix

Amplification des séquences recombinées = Amplification of recombined sequences

e) Clonage des séquences recombinées = Cloning of recombined sequences

CRIBLAGE des séquences recombinées = SCREENING of recombined sequences

Famille de séquences polynucléotidiques recombinées = Family of recombined polynucleotide sequences

RETOUR EVENTUEL A L'ETAPE a/ = POSSIBLE RETURN TO STAGE a/

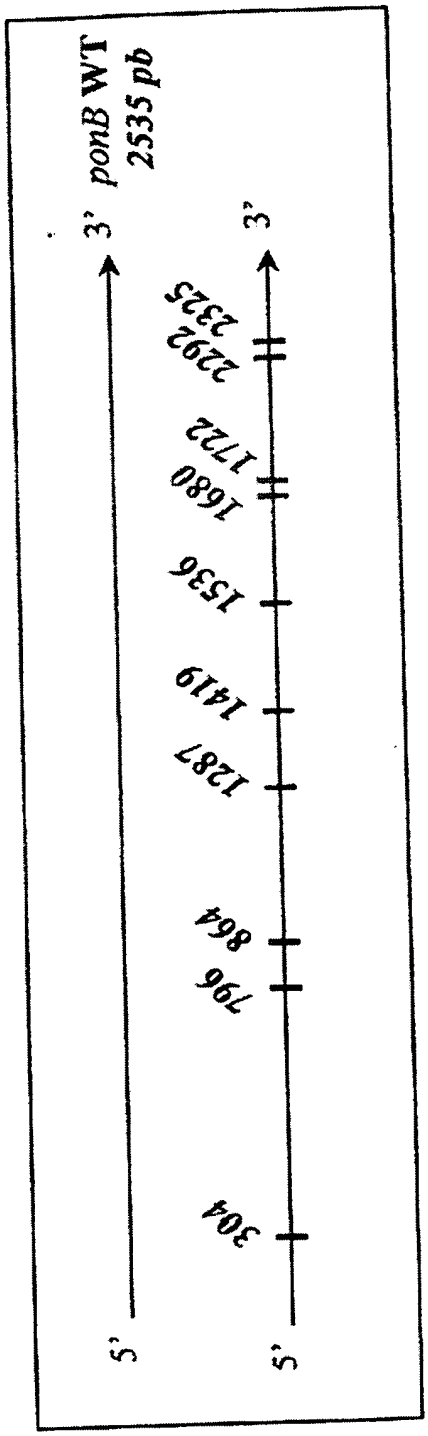


Figure 3: Position des dix zones de mutations (sites *Pvu* II et *Pst* I)

[Key to Figure 3:]

Position des dix zones de mutations (sites *Pvu* II et *Pst* I) = Position of ten mutation zones (*Pvu* II and *Pst* I sites)

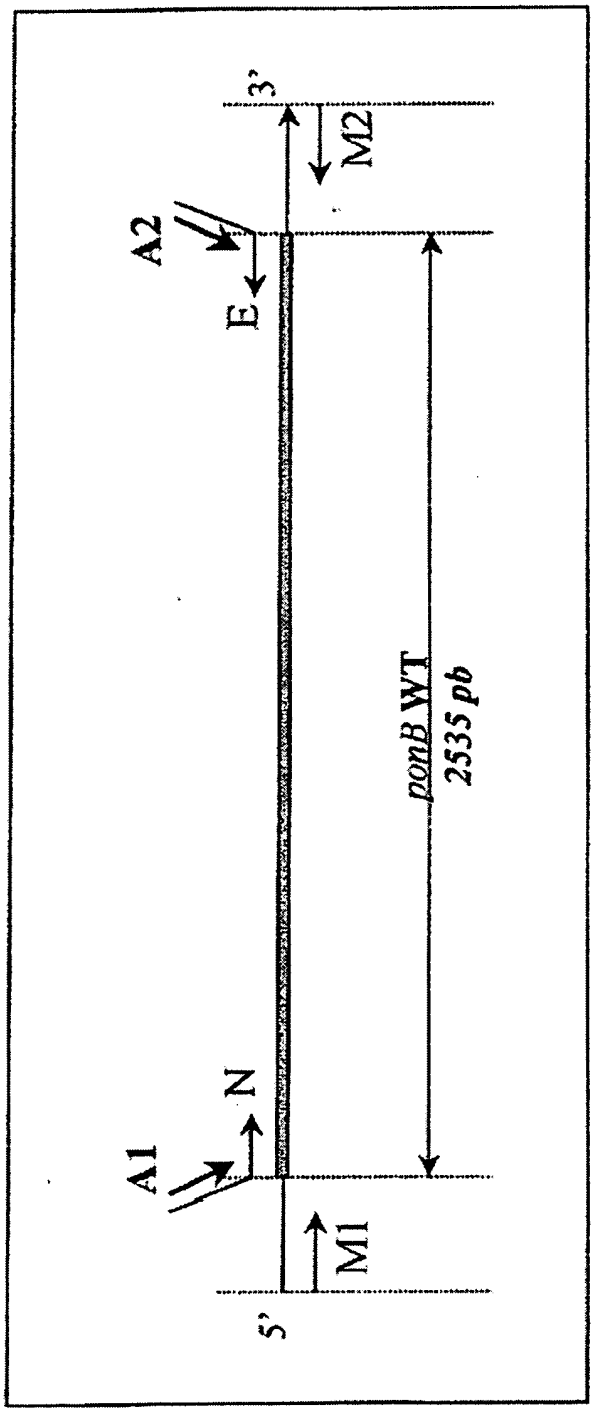


Figure 4 : Position des amorces utilisées par rapport à la séquence du gène *ponB*

[Key to Figure 4:]

Position des amorces utilisées par rapport à la séquence du gène *ponB* = Position of primers used relative to the sequence of the *ponB* gene

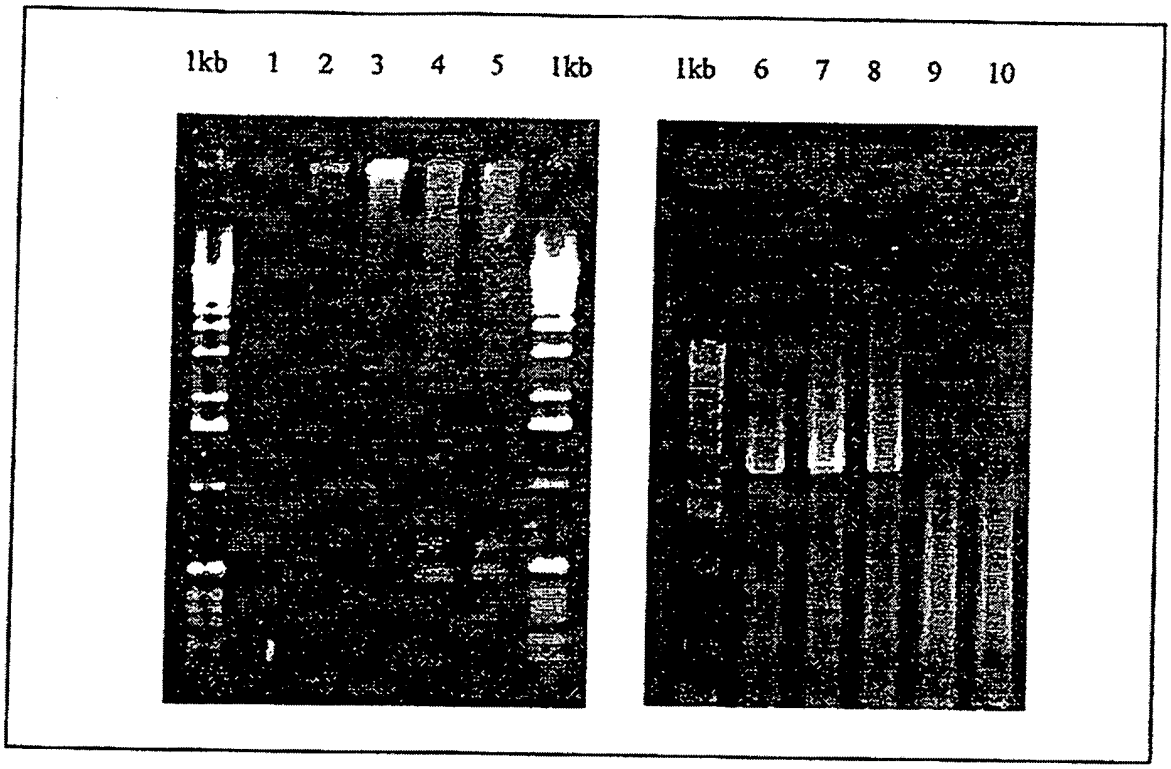


Figure 5: Migration of the RLR reactions and the PCR amplifications of these reactions

Tracks:	*1/ RLR 1*	*6/ PCR RLR 1*
	2/ RLR 2	*7/ PCR RLR 2*
	3/ RLR 3	*8/ PCR RLR 3*
	4/ RLR 4	*9/ PCR RLR 4*
	5/ RLR Control	*10/ RLR Control PCR*

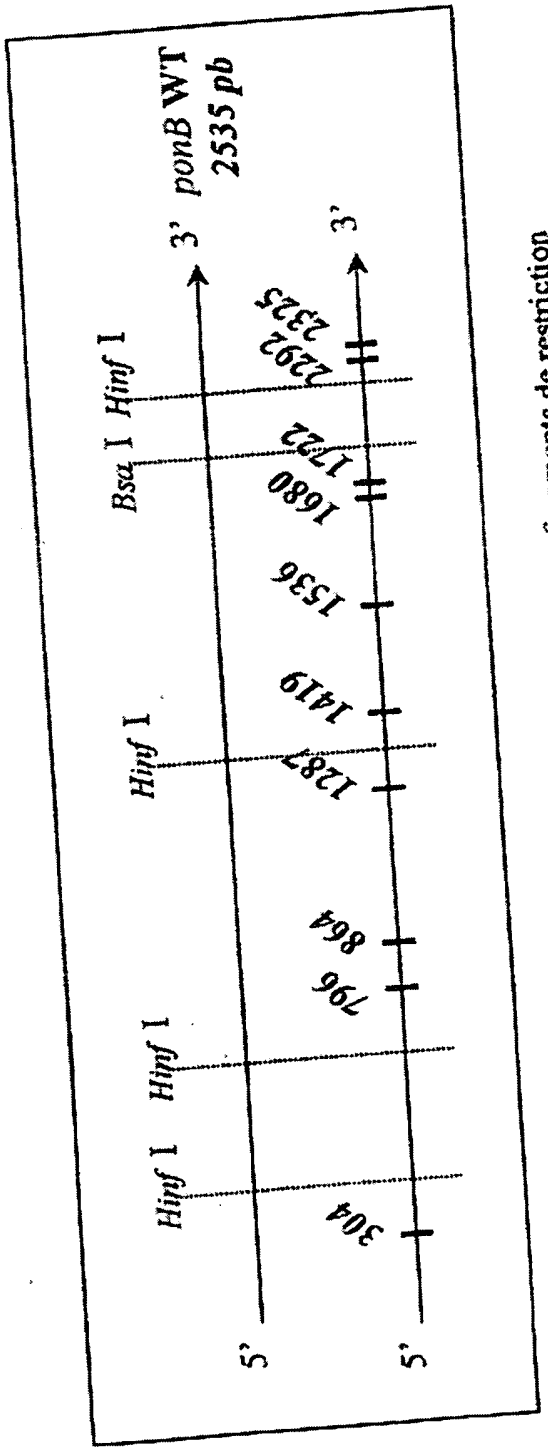


Figure 6 : Position des mutations par rapport aux fragments de restriction

[Key to Figure 6:]

Position des mutations par rapport aux fragments de restriction = Position of Mutations Relative to Restriction Fragments